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13. ABSTRACT (Maximum 200 words)

Based on the data we have generated in the first six months of the project, we believe we have exceeded the milestones for this project. Initial genetic constructs for the Herceptin IgG human antibody were made and transformed into *Trichoderma reesei*. These strains were screened for antibody production in shake flasks. A few of the strains were tested at the 14-liter fermentation scale. We have been able to produce over 3 g/L of full length IgG as determined by ELISA. We have produced 0.7 g/L of a non-glycosylated variant of the Herceptin IgG human antibody at 14L scale. A number of different genetic constructs have also been made to optimize the Kex2 cleavage site. Our best constructs show greater than 90% cleavage of the fusion proteins to products. Our data indicate that *Trichoderma* reesei is an efficient host for the production of antibodies.

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Final Progress Report

Develop Systems for Manufacturing 100,000,000 Doses of an Emergency Pharmaceutical (E.G. Vaccine or Monoclonal Antibody) Within 2 Months of Product Identification.

Contract Number: W911NF-05-C-0072

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Statement of the problem studied

During the six months of the project, we focused on producing heavy and light chains of human IgG antibodies as fusion proteins in *Trichoderma reesei*. We optimized the cleavage site to achieve maximum secretion of the mature protein. We also studied the production of non-glycosylated antibody either by constructing non-glycosylated mutants or by *in situ* removal of glycosylation from the wild type antibody. We have also characterized the antibody for proper folding and activity.

Several groups have previously used filamentous fungi, especially *Trichoderma reesei* and *Aspergillus* species, as hosts for mammalian protein production. At Genencor, we have developed strains of *Aspergillus niger* var. awamori, which demonstrate improved secretion of foreign proteins (Ward et al., 1993). Typically, the desired mammalian protein is produced as a fusion to the C-terminus of a highly secreted native protein, such as *A. niger* glucoamylase (GA) or T. *reesei* cellobiohydrolase I (CBHI), because this strategy can increase titers significantly compared to production as a non-fusion protein (Ward et al., 1990) (Figure 1). A cleavage site (Lys Arg "KR") recognized by the Golgi serine proteinase Kex2 (Jalving et al., 2000) is often used to allow release of the authentic mammalian protein during the secretion process (reviewed in Gouka et al., 1997) (Figure 2). This approach has been used by Frenken et al. (1998) for the production of an scFv in *A. niger*. Work in T. *reesei* has demonstrated production and assembly of a Fab fragment. In this case, the heavy chain (Fd) was expressed as a fusion with the native secreted CBHI while the light chain was not expressed as a fusion protein (Nyyssonen et al., 1993). Titers of up to 0.15g/L of secreted Fab were observed. Genencor recently demonstrated that an IgG1 antibody and a Fab' can be produced efficiently in *A. niger* with secreted titers of up to 0.9 g/L of the full-length antibody (Ward et al., 2004).

Unlike antibody fragments, full-length IgG1 molecules include an N-linked glycosylation site in the Fc portion of the heavy chain. Production of the antibody in alternative hosts can lead to lack of glycosylation in bacteria or altered glycosylation in eukaryotic hosts. It has been shown that the structure of the glycan can be important with respect to pharmacokinetics (Wright and Morrison, 1994), although non-glycosylated IgG1 has a serum half-life similar to wild-type IgG1 (Tao and Morrison, 1989). Therapy with a monoclonal antibody may depend on effector function that resides in the Fc domain. Recruitment of cytotoxic cells or complement activation may require appropriate glycosylation of the Fc region for optimum interaction with FcgR receptors or C1q (Lund et al., 1996; Jefferis et al., 1998). Alternatively, antigen binding alone may be sufficient for some therapeutic purposes and effector functions may actually be undesirable. In these cases, a full-length antibody may still be preferred to benefit from the long serum half-life that results from the interaction of the Fc domain with FcRn, the IgG salvage receptor (Ghetie et al., 1996). However, glycosylation does not appear to be necessary for antibody interaction with FcRn, and glycan structure may also prove irrelevant for this interaction.

Summary of the most important results

Based on the data we have generated in the first six months of the project, we believe we have exceeded the milestones for this project. Initial genetic constructs for the Herceptin IgG human antibody were made and transformed into *Trichoderma reesei*. These strains were screened for antibody production in shake flasks. A few of the strains were tested at the 14-liter fermentation scale. We have been able to produce over 3 g/L of full length IgG as determined by ELISA. We have produced 0.7g/L of a non-glycosylated variant of the Herceptin IgG human antibody at 14L scale. A number of different genetic constructs have also been made to optimize the Kex2 cleavage site. Our best constructs show greater than 90% cleavage of the fusion proteins to products. Our data indicate that *Trichoderma reesei* is an efficient host for the production of antibodies.

Production of wild-type IgG antibodies in producing strain of *T. reesei*

The strategy to produce human IgG antibodies in *T. reesei* is based on construction of individual expression cassettes for both the heavy and light chain as protein fusions to the CBHI catalytic domain (Figure 3). Heavy and light chain sequences corresponding to wild-type Herceptin and also a variant Herceptin (allotype) were ordered from two Small Business Enterprises (GeneArt and DNA2.0) to increase chances of success. Each of these companies was asked to codon optimize the genes for *T. reesei*. Initially CBHI-light chain fusions were constructed and tested for expression in shake flasks. Codon optimized genes expressed better than the natural gene (Figure 4). These studies showed that the gene from GeneArt expressed at higher levels than the DNA2.0 gene (Table 1). The GeneArt light chain sequence also appeared to be cut into a smaller molecular weight product than the DNA 2.0 constructs (Figure 5). Examination of the gene sequences used for antibody production showed that there was a potential Kex2 site in light chain sequences used by GeneArt, which was not present in the DNA2.0 gene. This led us to change the “KR” amino acid sequence in the GeneArt gene to the amino acids, “TR”. There was also an internal “KR” amino acid sequence found in the heavy chain. This heavy chain “KR” sequence was also changed to the amino acids, “TR”. The best production constructs were then combined and used with heavy chain constructs to produce full-length complete antibody. Combining the best gene sequences (GeneArt), optimized Kex2 site, and removal of internal Kex2 sites (TR mutants) resulted in a strain (17-43), which was able to produce 0.17g/L in shake flasks and over 3 g/L in 14 L fermentation (Figure 6). Analysis of the samples taken over the course of the fermentation showed that a percentage of the heavy chain being produced at later time points was cleaved in the fermentation broth (Figure 7). This proteolysis is likely limiting the amount of total antibody that we can produce in this strain.

Production of non-glycosylated IgG

<u>Construction of non-glycosylated IgG mutants</u>

The Herceptin IgG antibody contains a single N-linked glycosylation site (NXT) at position 297. *T. reesei* glycosylation differs from human glycosylation. Since the glycosylation may affect the function of the antibody, we introduced mutations in the

heavy chain sequence to remove the single N-linked site. A number of different mutations were created which changed one of the amino acids at the N-linked glycosylation site. (N297D, S, Q and T299V, A, R). The mutants were screened in shake flasks for expression levels of the antibody. The T299V change was found to produce the highest levels of antibody. This strain was tested in a 14L fermentation run and was found to produce 0.7g/L of antibody (Figure 8).

In situ removal of glycosylation of the wild-type anitbody heavy chains

An alternate strategy to produce deglycosylated antibodies is to remove the glycosylation by enzymatic cleavage. *T. reesei* produces an enzyme (endoT) which removes the sugars from the N-linked glycosylation sites of proteins. The endoT gene from *T. reesei* has been cloned and expressed in *T. reesei* under the CBHI promoter. Several clones were screened at the shake flask level and shown to produce endoT protein.

Fusion protein cleavage

The CBHI-antibody fusions were designed with a Kex2 site linking the two proteins. The Kex2 protease is naturally found in the Golgi of *T. reesei* where it processes protein domains. Several different Kex2 site variants were constructed and tested using the CBHI – light chain antibody fusions to determine the best sequences for cleavage optimization. The initial Kex2 site used in the CBHI-light chain fusion was "KRGGG" which was used in the original *A. niger* constructs. This was found to be cleaved at about 50% efficiency. This site also has the disadvantage of leaving three glycine amino acids at the N-terminal of the light chain. Moving the three glycine residues in front of the KR cleavage site (GGGKR) reduced the cleavage to less than 10%. Optimizing the Kex2 site resulted in nearly 100% cleavage of the fusion protein, in addition this construct left the native N-terminal of the light chain (Figure 9). N-terminal sequencing of the different clones confirmed that cleavage occurs at the expected sites. The optimized Kex2 site was used in all CBHI-antibody fusion proteins. Additional work was performed to determine which of the residues in the optimized sequence are required for the improved cleavage, and to screen for better cleavage site sequences (Figure 10). A patent application will be filed on the optimized Kex2 site.

14 L Fermentations for Strain Evaluations

14L fermentations were run to test the various Herceptin antibody constructs. Strains were fermented in the standard *T. reesei* process using a glucose sophorose feed (40% glucose, ~8g/L sophorose) fed at a specific rate of 0.025 g/g DCW/hr at pH 4.8 and a production temperature of 28^0C. *T. reesei* fermentations normally are run for 200hrs. Samples were taken at various time points and analyzed for antibody production.

Analysis of Antibody produced

Previous work on antibody production in the *Apergillus niger* fungal system showed that the secreted heavy and light chains were properly folded and were active. (Ward et al., 2004). We screened the expression of our constructs in *T. reesei* using immunology-

based methods. Initial screening was preformed using an ELISA assay. The ELISA used two human IgG specific antibodies. The IgG was captured with an IgG FC specific antibody (Sigma I2136 Goat anti-human IgG FC specific). The IgG was detected using a Light chain specific antibody peroxidase conjugate (Sigma A7164 Goat anti-human Kappa LC Horseradish peroxidase). Using this method, only the IgG molecule, which contains both a heavy and a light chain, was detected.

The biological functionality of the antibodies produced was confirmed by testing in a cell proliferation assay using Herceptin as a control (Figure 11). The *T. reesei* antibodies preformed similar to the Herceptin controls. (Ward, et al 2004).

Key issues for moving forward

Our data suggests we are producing much greater amounts of antibodies in *T. reesei*, but they are being degraded during the fermentation. It appears that the heavy chain is being clipped near the hinge region, while the light chain continues to be produced at much higher levels (~10 g/L). This is currently limiting production of the whole antibody. Identification and removal of the proteases responsible for this clipping should allow greater amounts of antibodies to be produced.

Listing of all publications and technical reports

None

Listing of all participating scientific personnel showing any advanced degrees

None

Report of Inventions (by title only)

Kex2 Cleavage Regions Of Recombinant Fusion Proteins

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Appendixes-Illustrations and Tables

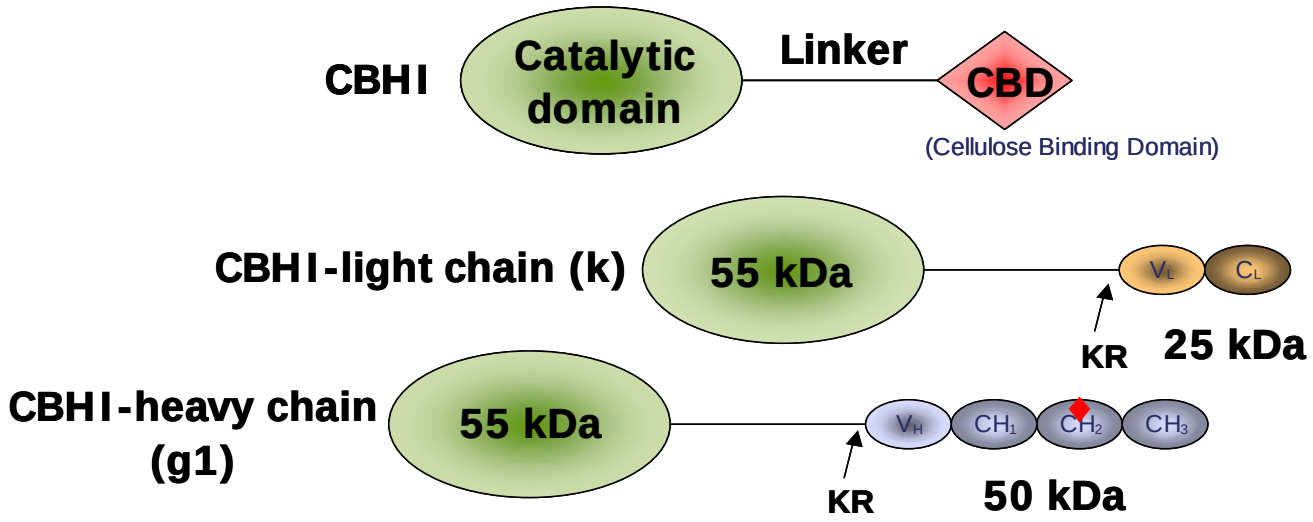

Figure 1. Strategy for Ab production in *Trichoderma*.

Produce both H and L chains fused to cellobiohydrolase 1 (CBHI) Core for improved secretion. Transform separate vector for each chain into the same cell. Include a Kex2 cleavage site (KR) for cleavage *in vivo.*

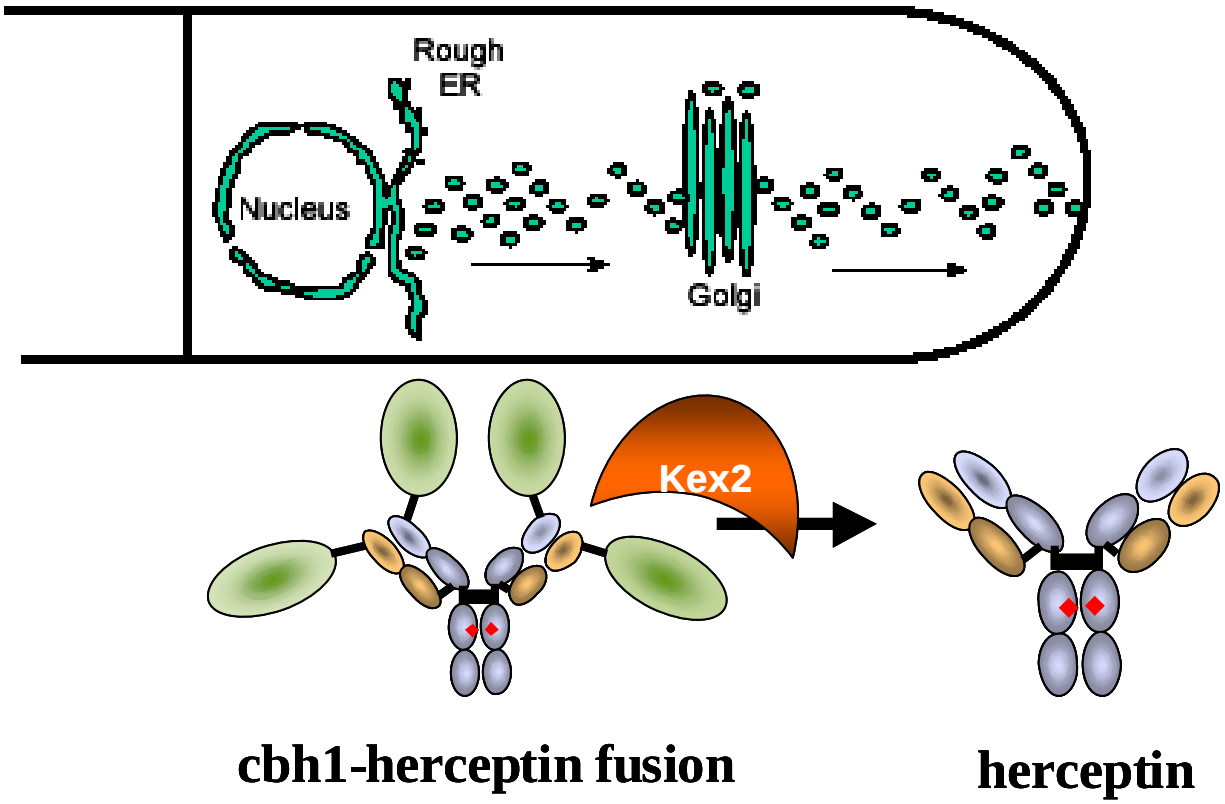

Figure 2. Predicted assembly and processing of Abs in *T. reesei*.

The CBH1-light chain and CBH1-heavy chain fusion proteins should assemble in the ER. Cleavage should occur later in the secretion pathway (Golgi)

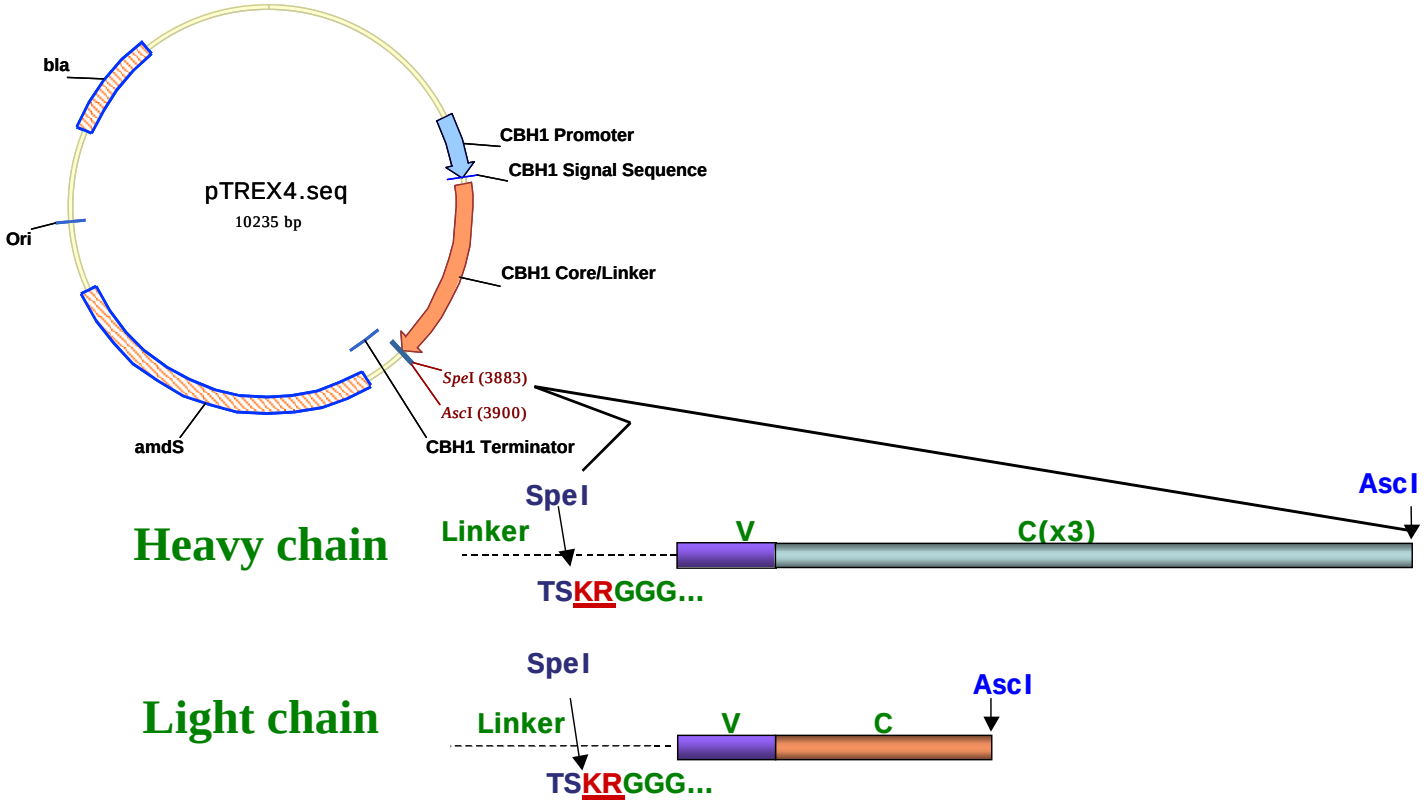

Figure 3. Design of Herceptin Heavy and Light chain expression constructions for *T. reesei*.

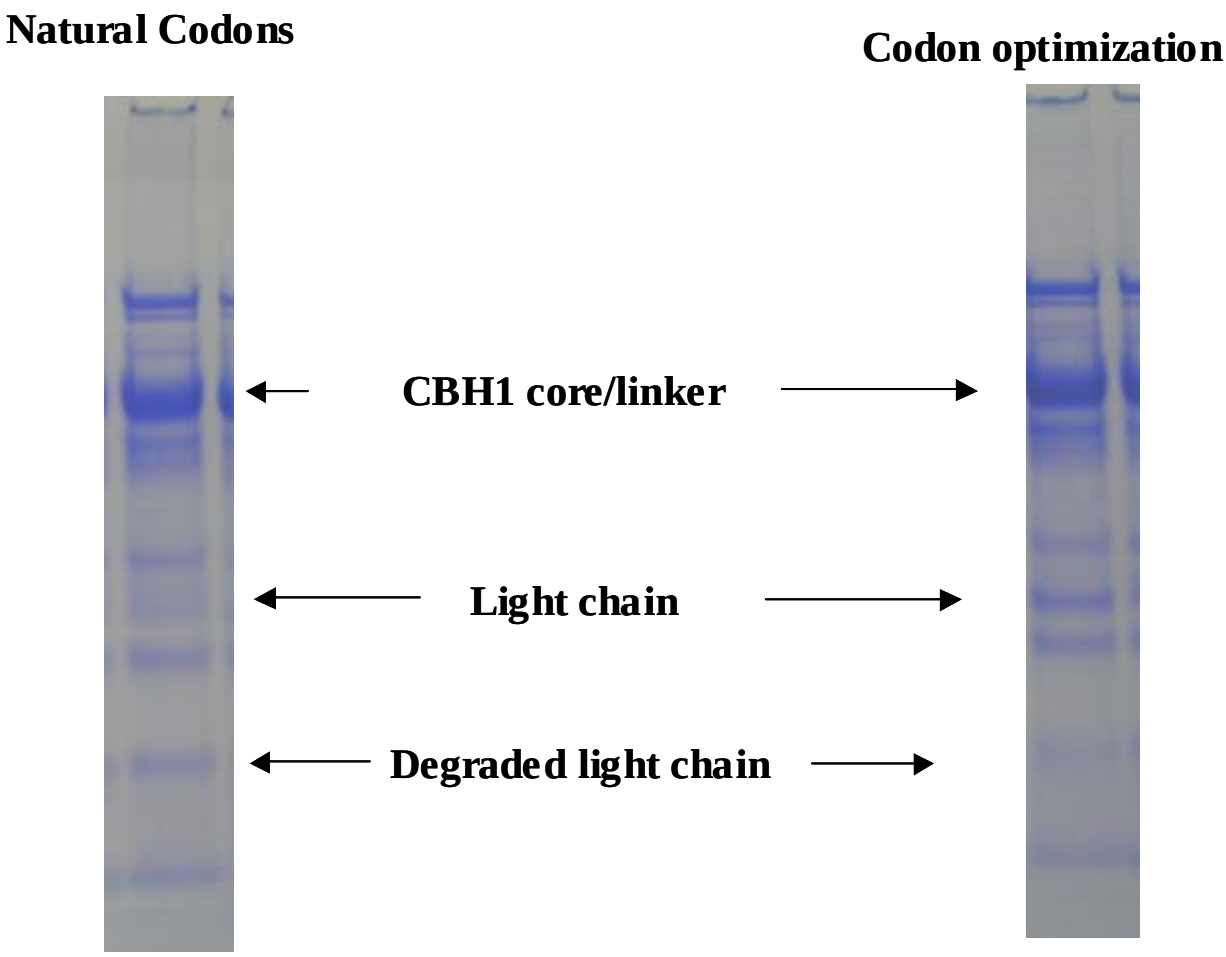

Figure 4. Codon optimization effects.

Comparison of expression of the CBHI-light chain fusion from a natural codon based gene and codon-optimized gene.

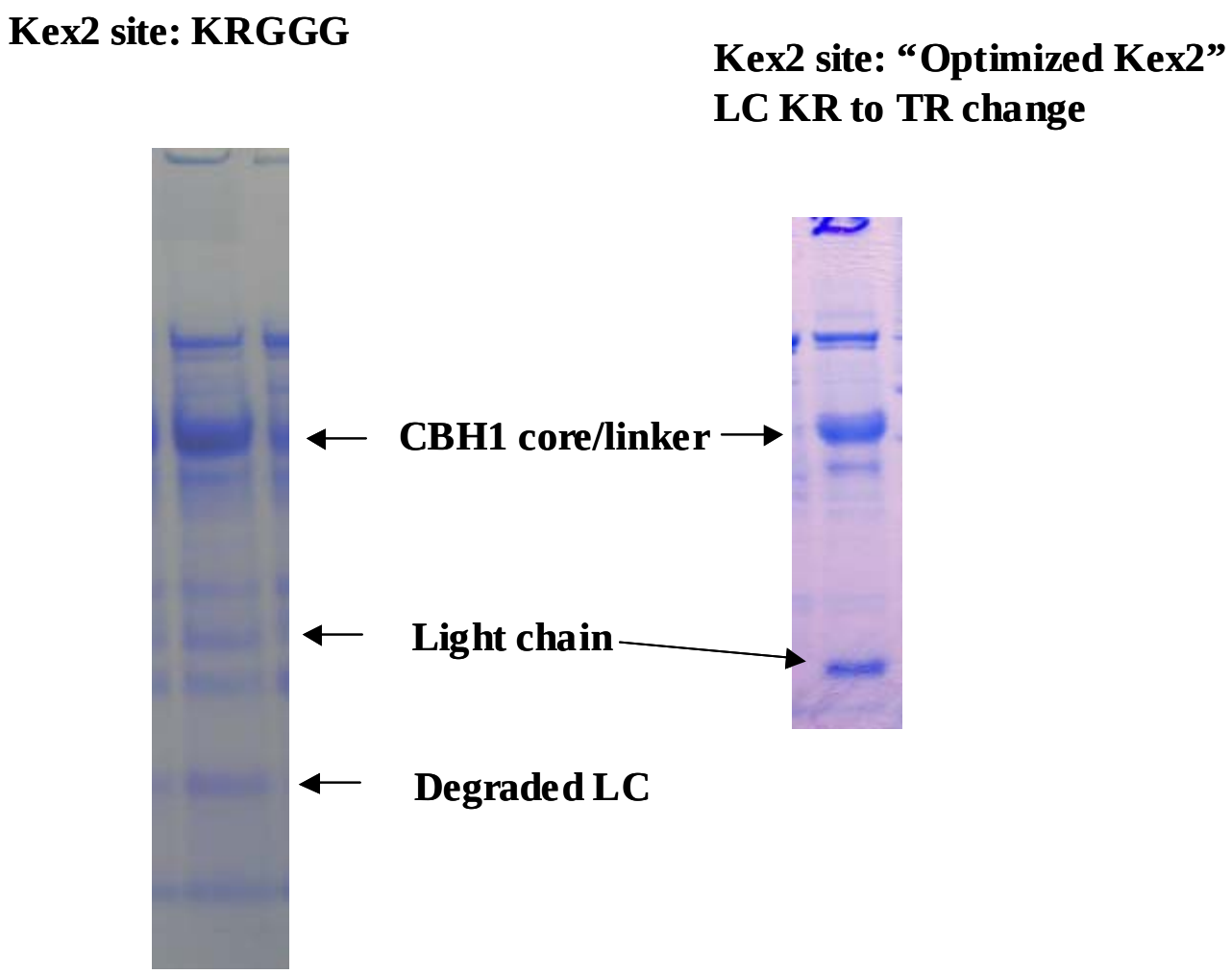

Figure 5. Protease at Internal Kex2 site in Light Chain

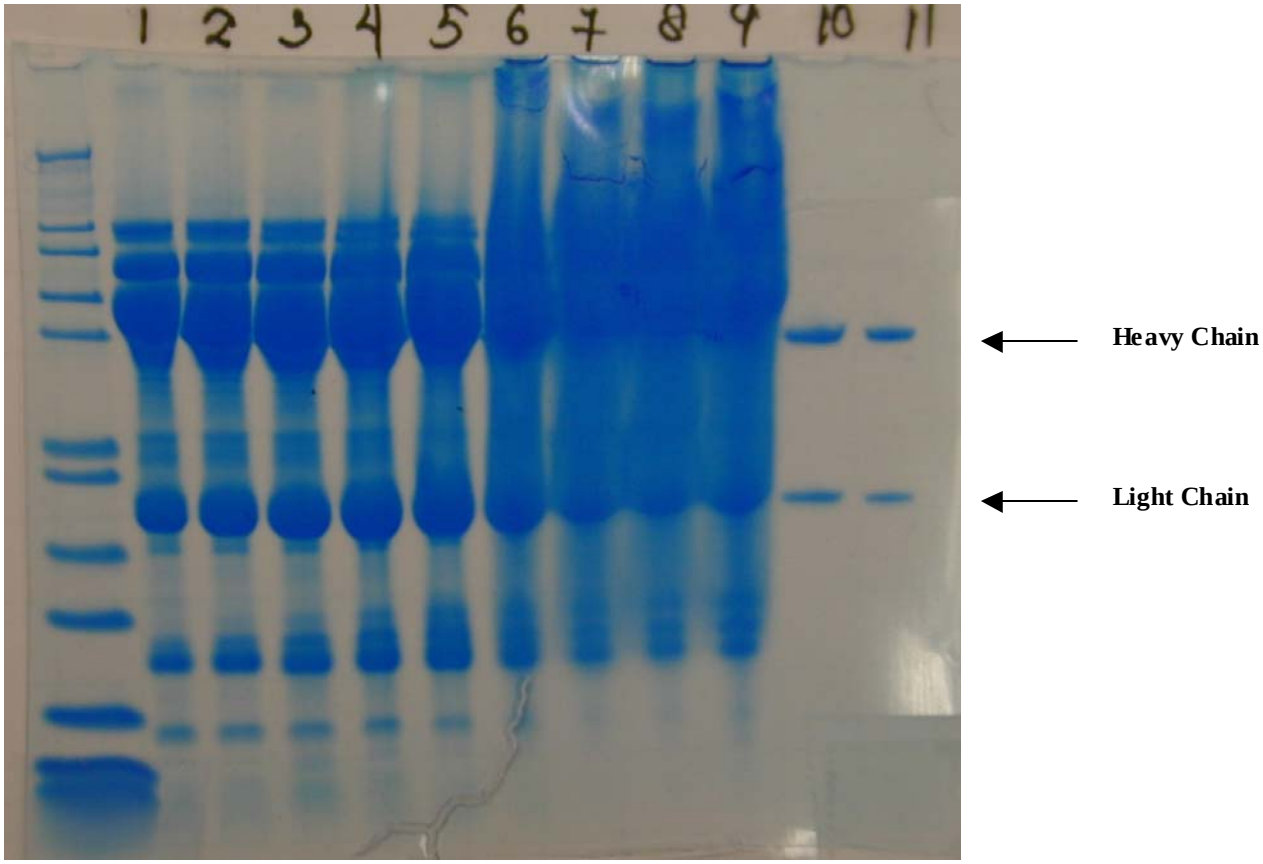

Figure 6. 14L Fermentation of Strain 17-43

Lanes 1-9 Time points from fermenter 66 hr, 79 hr, 91 hr, 103 hrs, 117 hrs, 156 hrs, 168 hrs, 180 hrs. Lanes 10- 11 IgG standards

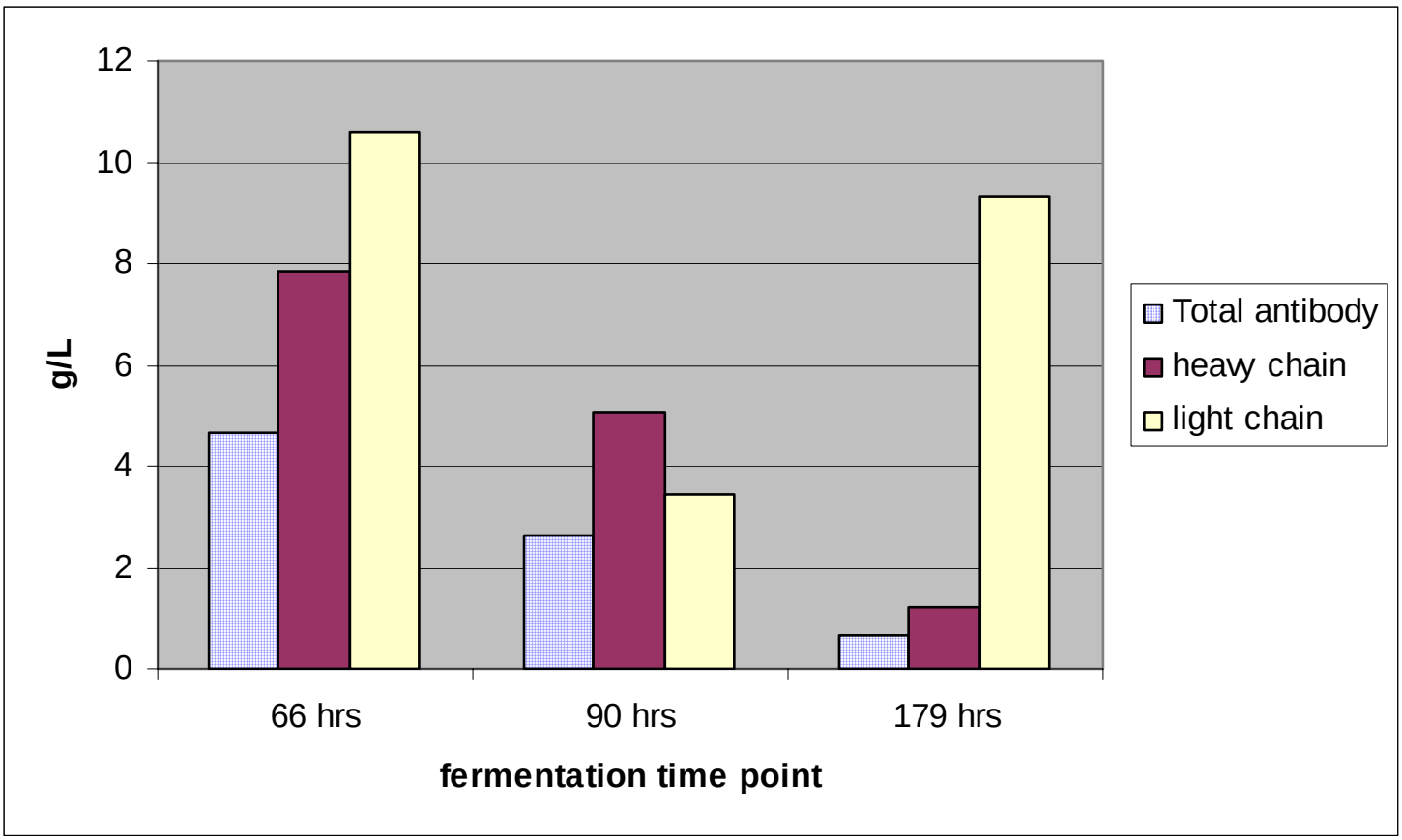

Figure 7. Ratio of total antibody to heavy and light chain found in 14 L fermentation of strain 17-43.

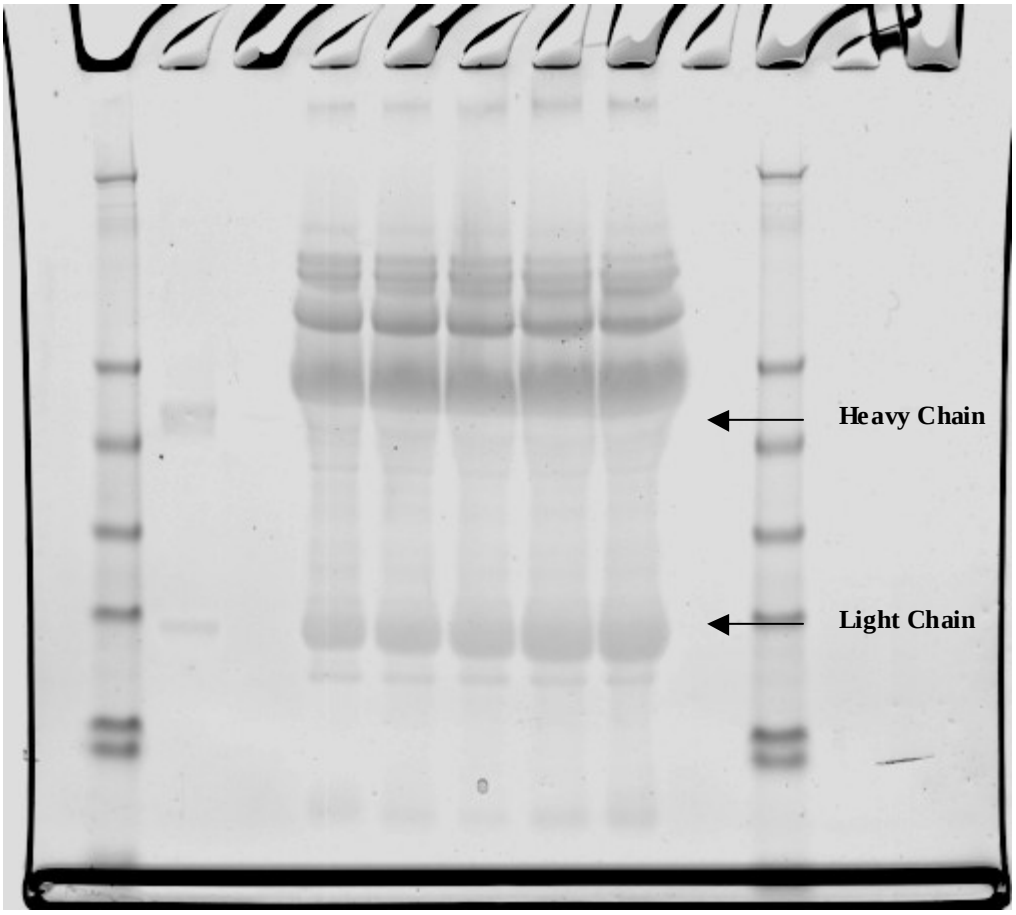

Figure 8. 14L Fermentation of non-glycosylated mutant T299V Strain 19-6

Lanes 3-7 Time points from fermenter 60 hr, 72 hr, 90 hr, 101 hrs, 120 hrs.
Lane 2 IgG standards Lanes 1, 8 MW Markers

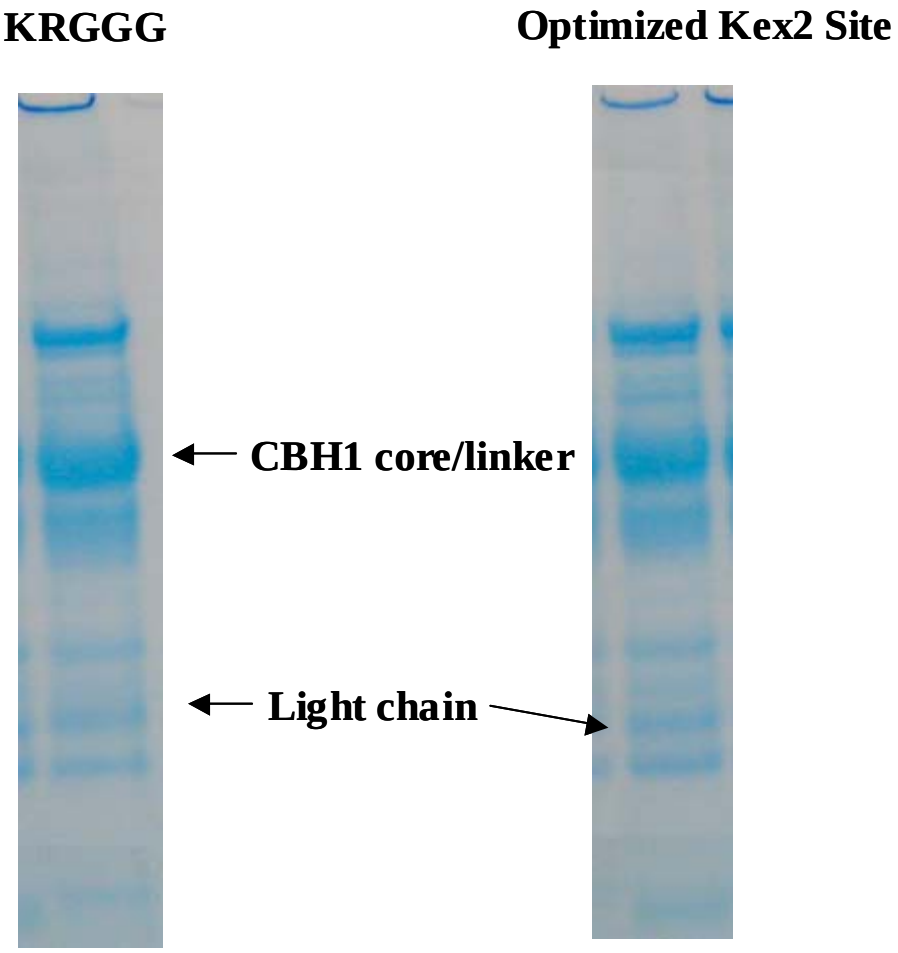

Figure 9. Effect of the Kex2 site region on Kex2 processing of the CBHI-light chain fusion protein.

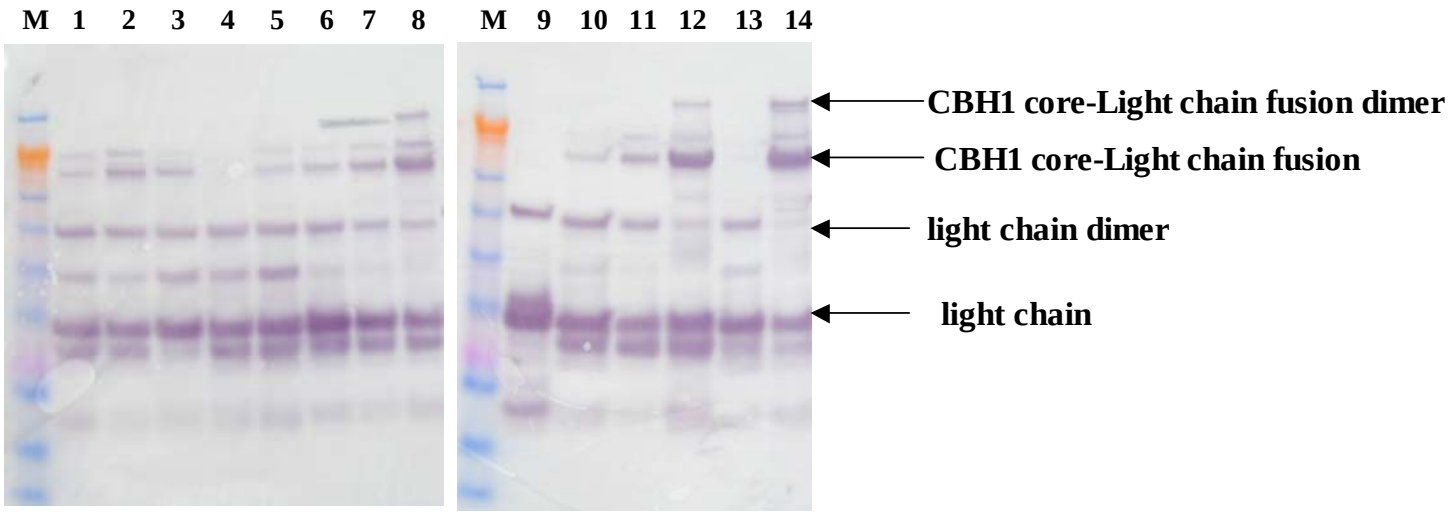

Figure 10. Western gel of variants in the optimized Kex2 site linking CBHI-light chain fusions.

Lanes: M-See Blue Plus 2 (molecular weigh marker), Lanes 1-14, variants of the optimized Kex2 site.

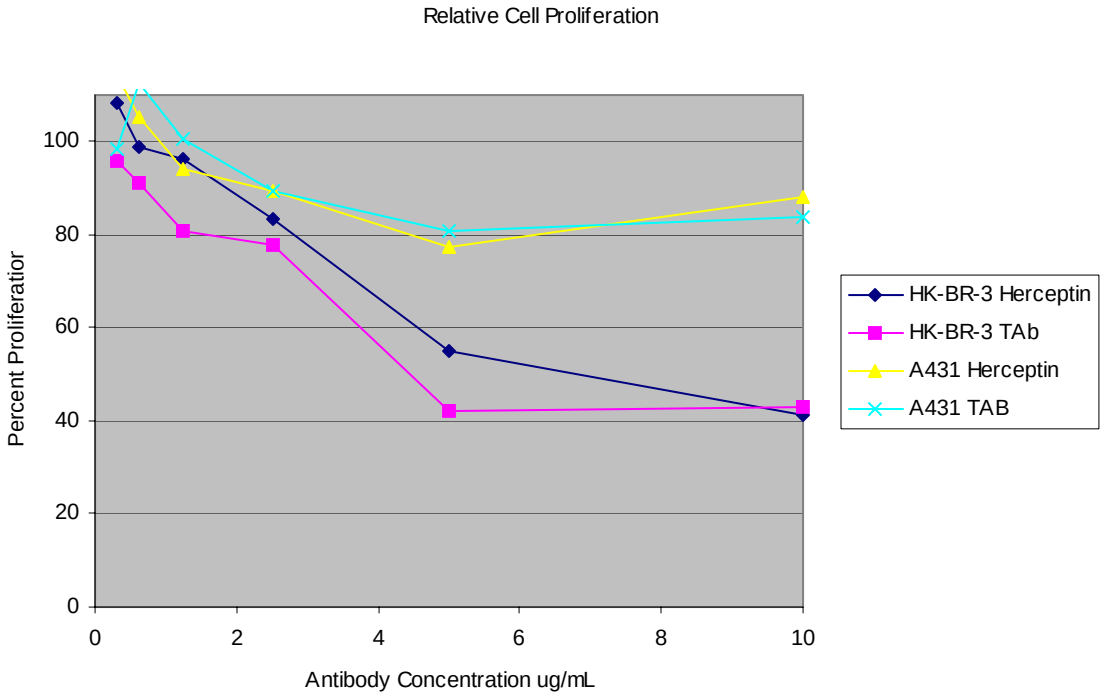

Figure 11. Cell Proliferation assay comparing *T. reesei* produced antibodies to a Herceptin control.

Table 1. Comparison of expression for the various strains in shakes flasks and 14 L fermentation.

Strain	**Shake Flask** **(best clone)** **(mg/L)**	**14L** **(mg/L)**
#3-6 codon opt DNA2.0, KEX2 site: KRGGG	0.7	4.8
#10-11 codon opt DNA2.0, KEX2 site: KRGGG, N->Q mutant at glycosylation heavy chain site.	-	1.8
#15-29 codon opt GeneArt, KEX2 site: KRGGG	15	210
#16-23 codon opt GeneArt, KEX2 site: Optimized Kex2, KR -> TR mutation in Light Chain	47	-
#17-43 codon opt GeneArt, KEX2 site: Optimized Kex2, KR -> TR mutation in Light Chain, KR -> TR mutation in Heavy Chain	170	3000
#18- codon opt GeneArt, KEX2 site: Optimized Kex2, KR -> TR mutation in Light Chain, KR -> TR mutation in Heavy Chain glycosylation site T->V	-	700